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Award Number: DAMD17-99-1-9531

TITLE: Modulation of Androgen-Induced Oxidative Stress Responses  
as a Chemopreventive Measure in Prostate Cancer

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REPORT DATE: September 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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<b>1. AGENCY USE ONLY (Leave blank)</b>			<b>2. REPORT DATE</b> September 2001		<b>3. REPORT TYPE AND DATES COVERED</b> Annual (16 Aug 99 - 15 Aug 01)	
<b>4. TITLE AND SUBTITLE</b> Modulation of Androgen-Induced Oxidative Stress Responses as a Chemopreventive Measure in Prostate Cancer			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9531			
<b>6. AUTHOR(S)</b> Dr. Todd A. Thompson						
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  University of Wisconsin-Madison Madison, Wisconsin 53706-1490  E-mail: thomps60@facstaff.wisc.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>			
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>			
<b>11. SUPPLEMENTARY NOTES</b>						
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Distribution authorized to U.S. Government agencies only (proprietary information, Sep 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				<b>12b. DISTRIBUTION CODE</b>		
<b>13. ABSTRACT (Maximum 200 Words)</b>  We have previously reported that androgens induce oxidative stress in the androgen-responsive LNCaP prostate carcinoma cell line. We hypothesize that androgen-induced changes in prostate redox status may contribute to prostate carcinogenesis. Chemopreventive measures may reduce the development of prostate cancer. This study addresses several molecular mechanisms implicated in cellular redox status. Intracellular calcium levels were increased by androgen exposure, which could be inhibited by vitamin E treatment. Activity of the redox sensitive transcription factors AP-1 and NF-κB were induced by androgen exposure in LNCaP cells, but not in the androgen-independent DU-145 cell line. AP-1 activity was also increased by vitamin E exposure in LNCaP and DU145 cells. Nitric oxide production was found to be an unlikely mediator of androgen-induced oxidative stress in LNCaP cells. The antiapoptotic Bcl-2 whole cell protein levels were decreased by exposure to 1 nM R1881 and 20 μM α-tocopherol succinate whereas mitochondrial Bcl-2 protein levels were found to be increased under these conditions. The mitochondrial Bax protein levels were found to be decreased by exposure to 1 nM R1881 and 20 μM α-tocopherol succinate. These results may provide valuable information for pathways to investigate as targets for chemopreventive measures in prostate carcinogenesis.						
<b>14. SUBJECT TERMS</b> Prostate Cancer				<b>15. NUMBER OF PAGES</b> 21		
				<b>16. PRICE CODE</b>		
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited			

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## Introduction

Prostate cancer is a disease whose incidence markedly increases with age. Many factors may contribute to both the initiation and progression of prostate cancer. Androgens are known to play a role in prostate cancer development. For example, eunuchs, men whose androgen levels have been reduced through castration, do not develop prostate cancer. Yet, the role of androgens in prostate carcinogenesis is poorly understood. We have previously shown that exposure of the LNCaP androgen-responsive prostate carcinoma cell line to physiologic levels of androgen can lead to the production of reactive oxygen species (ROS). ROS have been implicated as etiological factors in aging and may have a role in the development of cancer. We hypothesize that androgen exposure may alter the redox status of the prostate gland in a way which contributes to the development of prostate cancer. Our initial efforts in addressing our hypothesis have focused on understanding the mechanism(s) through which androgens lead to the development of ROS in prostate cells. This study addresses several molecular mechanisms that have been implicated in cellular redox status changes including cellular calcium homeostasis, redox-sensitive transcription factor activation, nitric oxide generation, and the role of redox-sensitive apoptotic factors. Epidemiological studies on dietary antioxidants, including vitamin E, have reported reduced prostate cancer incidences (1). Importantly, these studies suggest that chemopreventive measures which target cellular redox pathways may be effective in reducing the age-related onset of prostate cancer. By determining which pathways contribute to changes in androgen-induced cellular redox in prostate cells we hope to identify pathways that may serve as targets for chemopreventive measures in prostate carcinogenesis.

**Body**

We have shown that androgens induce an oxidative stress in the androgen-responsive LNCaP prostate carcinoma cell line (2). This may serve a useful model for investigating modes of antioxidant action in prostate carcinogenesis. However, the mechanism of androgen-induced oxidative stress in LNCAP cells is not fully understood. The purpose of this study was to investigate mechanisms of reactive oxygen species (ROS) production in the LNCaP human prostate carcinoma cell line that occurs on exposure to high physiologic levels of androgen (e.g. 1.0 nM R1881) and how exposure to vitamin E may modulate ROS production in this system. The synthetic, metabolically stable androgen R1881 was used in these studies. ROS are not produced following exposure to a growth proliferative dose of 0.1 nM R1881 in LNCaP cells or in androgen-independent cells, such as the DU145 human prostate carcinoma cell line, after androgen exposure. More specifically, this study addresses several molecular mechanisms that have been implicated in cellular redox status changes, including cellular calcium homeostasis, redox-sensitive transcription factor activation, nitric oxide generation, and redox-sensitive apoptotic factors. For the purposes of this report, the data is presented as Tasks as listed in the original Statement Of Work.

Vitamin E (i.e.  $\alpha$ -tocopherol) is poorly water-soluble. Also, different forms of  $\alpha$ -tocopherol have different water solubilities. In these studies  $\alpha$ -tocopherol succinate (ATS), which is converted to  $\alpha$ -tocopherol by cellular esterases and is moderately water soluble, was used. The importance of controlling the  $\alpha$ -tocopherol levels in preparations was realized due to the heterogeneity of responses observed by different investigators. For example, our original studies reported that 500  $\mu$ M concentrations of ATS were needed to abrogate androgen-induced ROS in LNCaP cells (2). In contrast, using a different method of reagent preparation, we found that 10 to 20  $\mu$ M concentrations of ATS were sufficient to reduce androgen-induced ROS in LNCaP cells (Appendix 1). Twenty  $\mu$ M ATS produced a significant reduction in ROS without producing cell death (data not shown) and was therefore the concentration of ATS used in most of these studies. A high performance liquid chromatography method was developed to standardize the ATS/media preparations used in these studies. Using this method to determine ATS levels has resulted in consistent observations between studies in our laboratory.

The purpose of Task 1 was to determine whether or not changes in intracellular calcium levels occur after androgen exposure and ATS treatment. The methods used to study calcium homeostasis in prostate carcinoma cells were similar to those originally developed for the Jurkat cell line (3). The addition of 1.0 nM R1881 for 96 hours produced a significant increase in intracellular calcium levels, while calcium was not elevated by exposure to 0.1 nM R1881 (Appendix 2A). Interestingly, the co-administration of 20  $\mu$ M ATS inhibited the increase in intracellular calcium levels observed after exposure to 1.0 nM R1881 (Appendix 2A). Although 20  $\mu$ M ATS alone did not significantly change calcium levels in LNCaP cells, intracellular calcium levels were decreased in DU145 cells after ATS exposure (Appendix 2B). The increase in intracellular calcium after exposure to 1.0 nM R1881 in LNCaP cells may reflect the development of a more differentiated prostate phenotype. This response may be secondary to ROS generation since the addition of vitamin E significantly inhibited the increase in calcium observed following androgen exposure.

We previously reported that AP-1 and NF- $\kappa$ B response element binding is increased after androgen exposure in LNCaP cells and this effect is decreased by ATS treatment (2). The ability of

this binding to affect transcriptional activity has not yet been evaluated. In Task 2, I proposed to investigate the modulation of AP-1 and NF- $\kappa$ B response elements on transcriptional regulation in LNCaP cells after androgen exposure using AP-1 and NF- $\kappa$ B luciferase reporter constructs. These studies were initiated by performing transient transfection analysis of the NF- $\kappa$ B luciferase reporter construct in LNCaP and DU145 cells with and without androgen exposure. The NF- $\kappa$ B expression vector used in these studies, pNF $\kappa$ BTKluc, contains three NF- $\kappa$ B response elements that regulate the expression of the luciferase gene from a minimal thymidine kinase promoter. A control vector, pmutNF $\kappa$ BTKluc, in which the NF- $\kappa$ B response elements have been mutated, was also used in these studies. Exposure of LNCaP cells to 1.0 nM R1881 for 48 hours resulted in a 2.4-fold increase in luciferase activity compared to unexposed (F1C4) cells (Appendix 3A). The increase in luciferase activity due to NF- $\kappa$ B response element activation has been repeated twice and was found to be statistically significant. This result was specific to the NF- $\kappa$ B response elements as no statistically significant increases were observed under these conditions with the pmutNF $\kappa$ BTKluc construct (Appendix 3B). Furthermore, the increase in NF- $\kappa$ B activity was found to be androgen-dependent since no changes in luciferase expression were observed in the androgen-independent DU145 cell line after androgen exposure (Appendix 4).

In addition to increases in NF- $\kappa$ B response element binding, we have also reported that AP-1 response element binding is significantly increased in LNCaP cells after androgen exposure (2). For these studies, a luciferase reporter vector driven by seven AP-1 response elements was used. LNCaP cells exposed to 20  $\mu$ M ATS for 48 hours showed a 2.5-fold increase in luciferase expression (Appendix 5A). A 3.3-fold increase in AP-1 activity was observed after exposure to 1.0 nM R1881, but no increase was seen with 0.1 nM R1881 (Appendix 5A). Interestingly, AP-1 activity was synergistically increased by the co-administration of androgen and ATS. For example, a 6.1-fold increase in luciferase expression was found after exposure to both 0.1 nM R1881 and 20  $\mu$ M ATS. A synergistic 9.5-fold increase in AP-1 activity was found after exposure to both 1.0 nM R1881 and 20  $\mu$ M ATS (Appendix 5A). Therefore, although some androgen-induced responses in LNCaP cells, such as changes in calcium levels, are abrogated by ATS treatment, others, such as AP-1 activation, do not specifically alter androgen-induced effects and result in augmented responses. In support of this observation, AP-1 activity was increased over 5-fold in DU145 cells exposed to 20  $\mu$ M ATS (Appendix 5B). Thus, ATS is a significant modulator of AP-1 activity in LNCaP and DU145 cells that is independent of androgen effects on AP-1 activity.

Studies to assess androgen-induced ROS generation in our laboratory were performed using the peroxide/hydroxyl radical sensitive probe dichlorodihydrofluorescein diacetate (DCF). Nitric oxide (NO) has been reported to produce the same fluorescent changes in DCF as peroxide and hydroxyl radical (4). In addition, it was reported that androgen exposure significantly increases NO synthase expression in LNCaP cells (5). Since NO production would allow a very specific pathway to target redox changes in prostate cells it was proposed in Task 3 to investigate the possible role of NO in redox changes in androgen-exposed LNCaP cells. To initiate these studies, a dose-response to the non-specific NO synthase inhibitor N<sup>G</sup>-methyl-L-arginine (NMA) was performed in LNCaP cells after 1.0 nM R1881 exposure. No decrease in the production of ROS was observed after a 4 day treatment with up to 100  $\mu$ M NMA (Appendix 6). In addition, the presence of ROS was determined following short-term exposure (i.e. 1 hour) of NMA and no changes were observed in the androgen-induced production of ROS in LNCaP cells (data not shown). Thus, it is unlikely that

NO is the source of ROS that produces DCF fluorescence after androgen exposure in LNCaP cells and, thus, further studies to evaluate NO as a key mediator of androgen-induced oxidative stress in LNCaP were not pursued. However, we have continued our collaboration with the electron paramagnetic resonance (EPR) facility at the National Biomedical EPR Center as proposed in Task 3. In addition to using spin traps that are sensitive to NO production, which will help confirm our NMA studies, other spin traps (e.g.  $\alpha$ -phenyl-*tert*-butylnitrone and 5,5-dimethylpyrroline-*N*-oxide) that will help identify the specific ROS species that are generated from androgen-exposure in LNCaP cells are being used. Furthermore, following a suggestion from our collaborators at the National Biomedical EPR Center, studies are being performed to analyze ROS production in mitochondrial preparations as well as whole cell preparations. These studies are currently in progress. In addition to helping determine which ROS species are responsible for the changes in redox status, this will help determine the subcellular source of ROS that develop after androgen exposure in LNCaP cells. If these studies are successful in measuring androgen-induced ROS, then further studies will be performed to determine the effect of ATS exposure on ROS production in androgen exposed LNCaP cells using EPR.

Alterations in the Bcl-2 family of proteins have been observed following changes in cellular redox status. Bcl-2 proteins affect cellular apoptotic responses with some Bcl-2 family members being proapoptotic (e.g. Bax, Bak, Bad) and others acting to prevent apoptosis (e.g. Bcl-2, Bcl-x<sub>L</sub>). Androgens have been reported to modulate Bcl-2 protein levels in LNCaP cells (6). In Task 4, I proposed to investigate the modulation of Bcl-2 family members after androgen exposure in LNCaP cells and how these changes may be affected by ATS treatment. Interestingly, we have found that androgen exposure potentiates ATS-induced apoptotic responses in LNCaP cells (7). Exposure of LNCaP cells to 0.1 nM or 1.0 nM R1881 did not significantly change whole cell protein levels of Bax, Bak, or Bcl-2 as measured by immunoblot analysis (Appendix 7A and 7B). However, exposure of LNCaP cells to 20  $\mu$ M ATS for 4 days produced a decrease in whole cell Bcl-2 protein levels, which was also observed in cells treated with 0.1 and 1.0 nM R1881. The levels of Bax and Bcl-2 proteins in mitochondrial preparations were also significantly altered. For example, mitochondrial Bax protein levels were decreased and Bcl-2 protein levels were increased in LNCaP cells exposed to 1.0 nM R1881 and treated with 20  $\mu$ M ATS (Appendix 7A), which are changes typically associated with antiapoptotic conditions in cells. Yet, we consistently observed an increase in apoptotic cell death under these conditions. This paradoxical observation requires analysis of the remaining Bcl-2 family members in addition to a systematic evaluation of their role in ATS- and androgen-induced apoptosis before any conclusions can be drawn. Bax and Bcl-2 protein levels were not altered in DU145 cells exposed to androgen or treated with 20  $\mu$ M ATS (data not shown). Nor were Bak protein levels affected by androgen or ATS exposure in DU145 cells (Appendix 7B). To date, we have not found antibodies to Bad or Bcl-x<sub>L</sub> that produce acceptable immunoblots. In these experiments, no changes in actin levels, which were used for immunoblot normalization, were observed in either LNCaP or DU145 cells after androgen or ATS exposure (Appendix 7C).

Dr. George Wilding has been an exemplary mentor over the course of this fellowship. In addition to his guidance in performing the experiments in these studies, he has also provided me with a wealth of knowledge regarding clinical and translational aspects of prostate cancer. Over the last year, I was strongly encouraged to pursue my interests in prostate cancer research through attending a number of scientific meetings that included the 2001 American Association for Cancer

Unpublished data

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Research Annual Meeting and the Hormonal Carcinogenesis Gordon Research Conference. Results from the research encompassed by this fellowship were presented at both meetings. Because of the excellent guidance and encouragement provided by Dr. Wilding during my post-doctoral fellowship, I plan to continue my future studies as an independent investigator in prostate cancer research.

The successful completion of the Tasks, as presented in the original Statement of Work, from this study have greatly contributed to our understanding of the development of reactive oxygen species in LNCaP cells resulting from androgen exposure. In addition to the knowledge gained from the studies presented in this report, I have formed significant collaborations with other investigators on the University of Wisconsin campus interested in prostate cancer. I assist in coordinating our monthly Prostate Group meeting and a monthly meeting on Cellular Stress Responses. Also, I am currently teaching 3 lectures on prostate cancer for the U.W. Department of Pathology and 2 lectures on carcinogenesis for the U.W. Environmental Toxicology Center. In conjunction with other ongoing studies in Dr. Wilding's laboratory, the results from these investigations have provided valuable information for pathways to examine as targets for chemopreventive measures in prostate carcinogenesis. A manuscript from the results of these studies is currently in preparation.

### Key Research Accomplishments

- Task 1 - Determine intracellular calcium levels after androgen and vitamin E exposure
  - Intracellular calcium levels were increased after 1.0 nM R1881 exposure in LNCaP cells
  - ATS inhibited the R1881-induced calcium increase in LNCaP cells
  - ATS decreased intracellular calcium levels in DU145 cells
- Task 2 - Determine AP-1 and NF-κB transcription factor activation in either AP-1 or NF-κB luciferase reporter constructs
  - AP-1 luciferase reporter activity increased by ATS and androgen exposure in LNCaP cells
  - AP-1 luciferase reporter activity increased by ATS treatment in DU145 cells
  - NF-κB luciferase reporter construct activity was stimulated by androgen in LNCaP cells
  - NF-κB luciferase reporter construct activity was not stimulated by androgen in DU145 cells
- Task 3 - Measure nitric oxide levels in androgen exposed LNCaP cells
  - Studies performed with NMA suggest that androgens do not induce NO in LNCaP cells
- Task 4 - Measure Bcl-2 family members by immunoblotting
  - Whole cell Bcl-2 protein was decreased by 1.0 nM R1881 and 20 μM ATS in LNCaP cells
  - Mitochondrial Bcl-2 protein was decreased by 1.0 nM R1881 in LNCaP cells
  - Mitochondrial Bcl-2 protein was stabilized by 1.0 nM R1881 and 20 μM ATS in LNCaP cells
  - Whole cell Bak protein was not altered by androgen or ATS exposure in LNCaP or DU145 cells
  - Mitochondrial Bax protein was decreased by 1.0 nM R1881 and 20 μM ATS in LNCaP cells
  - Actin expression was not affected by androgen or ATS exposure in LNCaP or DU145 cells

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### **Reportable Outcomes**

- 1 poster was presented at the 2000 Annual AACR Meeting from this work
- 1 poster was presented at the 2001 Annual AACR Meeting from this work
- 1 poster was presented at the 2001 Hormonal Carcinogenesis Gordon Conference from this work
- 1 manuscript is in preparation from these studies
- No patents or licenses have been applied for or issued as a result of these studies
- No degrees have been supported by this award
- No unique cell lines, tissues, or serum has been generated from these studies
- No informatic data bases or animal models have resulted from these studies
- No funding has been applied for based on this award
- Dr. Thompson was promoted in September 2001 to Assistant Scientist at the University of Wisconsin in part from the efforts supported by this award

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## Conclusions

An increase in intracellular calcium levels was observed in LNCaP cells exposed to high, physiologically relevant androgen levels. Importantly, this response was inhibited by vitamin E exposure. If the role of vitamin E is primarily as an antioxidant, then these results suggest that increases in calcium levels in LNCaP cells after androgen exposure may be mediated by the production of reactive oxygen species. AP-1 activity was increased by high, physiological androgen exposure in LNCaP cells. Furthermore, increased AP-1 activity was observed following vitamin E treatment in both LNCaP and DU145 cells. The changes in AP-1 activity by vitamin E treatment were not expected. Our original hypothesis was that cellular changes induced by androgen exposure would be inhibited by antioxidant treatment. In contrast, androgen and vitamin E acted synergistically to increase AP-1 activity in LNCaP cells. Further studies are necessary to determine the significance of vitamin E-induced transcriptional changes in prostate carcinoma cells. In addition to the direct antioxidant activity of vitamin E, the transcriptional changes induced by vitamin E exposure may provide an indirect means of antioxidant activity. Modulation of transcriptional activity may also contribute the antiproliferative activity of vitamin E. Transcriptional changes in NF- $\kappa$ B activity may also play a role in androgen-induced oxidative stress. However, nitric oxide production does not appear to be a factor in androgen-induced oxidative stress in LNCaP cells. Modulation of Bcl-2 family members may also contribute to the changes observed following androgen exposure in LNCaP cells. Importantly, androgen-induced changes in Bcl-2 family members were modulated by vitamin E exposure. Since many of the Bcl-2 family members are believed to be active in the mitochondria, it is likely that vitamin E effects may be reflected in the changes in Bcl-2 member protein levels in this subcellular organelle. This is supported by the changes in Bcl-2 and Bax protein levels observed in mitochondrial, but not whole cell lysates in this study. However, our current observations contrast with the generally accepted role of Bcl-2 and Bax as anti- and pro-apoptotic, respectively. That is, in the presence of 1.0 nM R1881 and 20  $\mu$ M ATS, the ratio of Bcl-2 to Bax was increased in the mitochondria; conditions that are typically associated with reduced cellular susceptibility to apoptosis. However, we observed increased apoptosis under these conditions. Furthermore, the ratio of Bcl-2 to Bax protein was decreased in mitochondrial extracts from 1.0 nM R1881 exposed LNCaP cells which is typically associated with an increased apoptotic response, whereas we find these cells to be highly viable. Therefore, further studies on other Bcl-2 members are being performed to determine their contribution to Bcl-2-mediated cell viability pathways. Significant opportunities in my study of prostate cancer have been afforded by the efforts supported by this award. For example, I have made significant progress in collaborations with other investigators interested in prostate cancer research on the University of Wisconsin campus, in the organization of prostate cancer study groups, and in prostate cancer education. Dr. George Wilding is an outstanding mentor for my studies in prostate cancer. A manuscript from the results of these studies is currently in preparation. The results from this award should provide valuable data for developing strategies in chemopreventive efforts of prostate cancer.

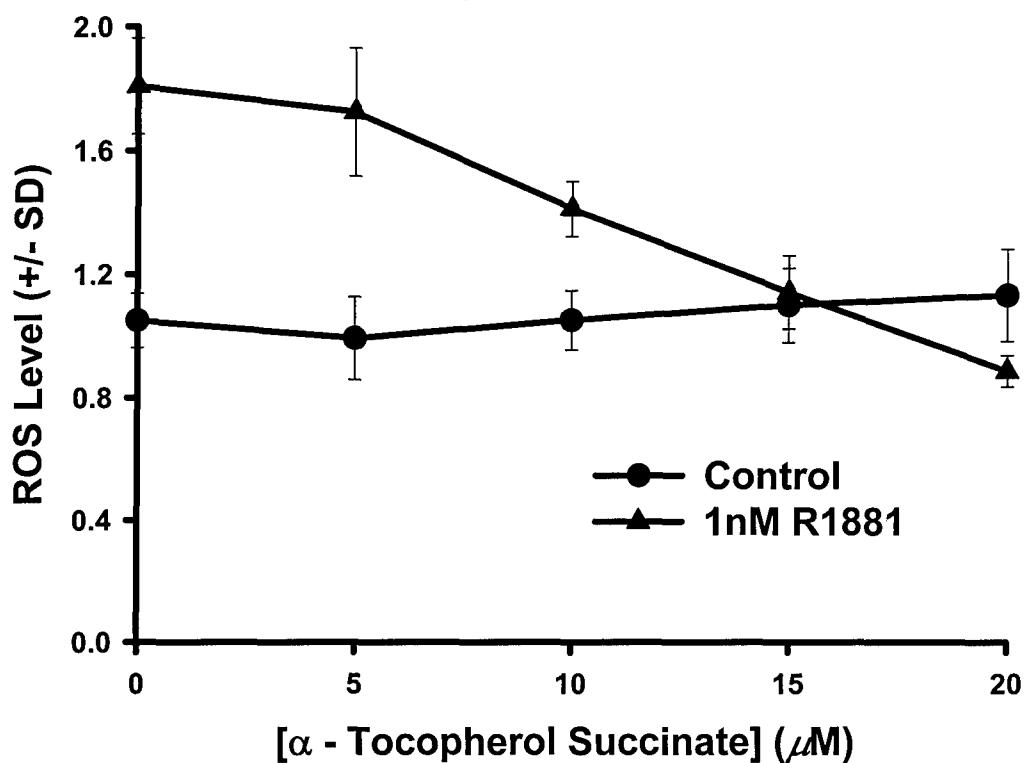
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## References

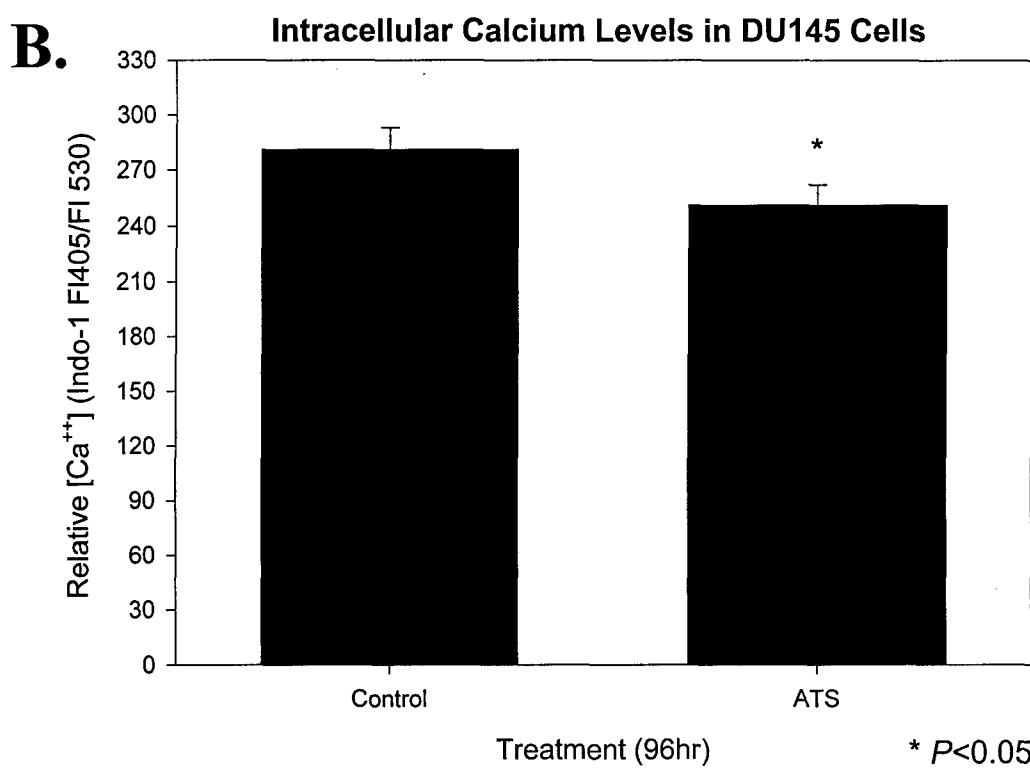
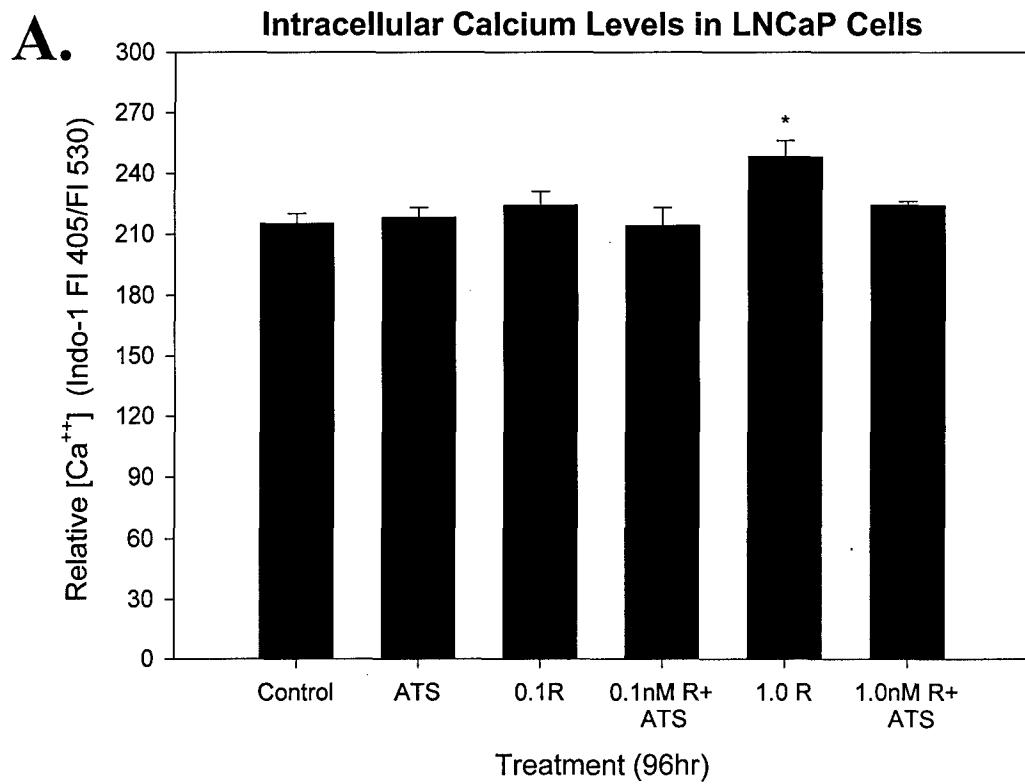
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# Appendix 1

## ROS response of LNCaP Cells to $\alpha$ -tocopherol succinate

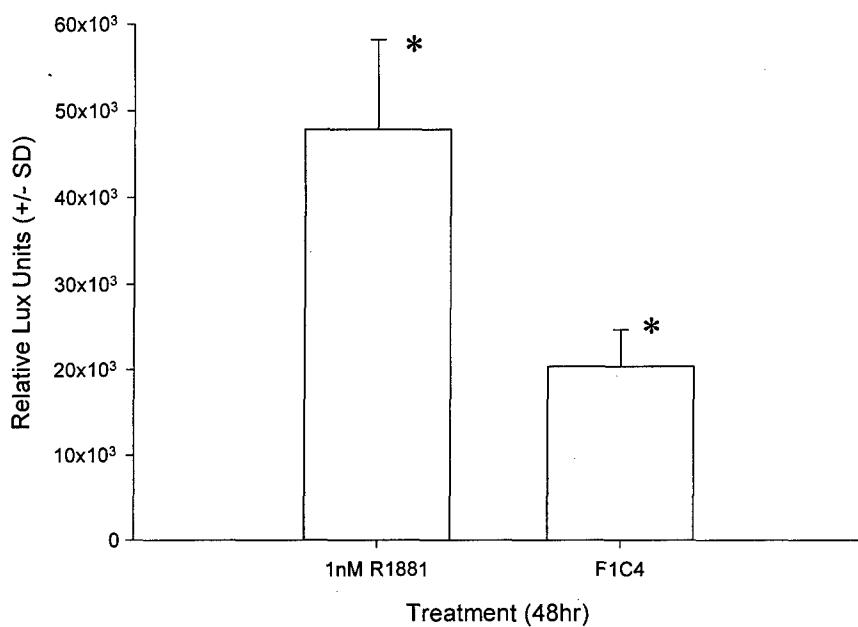


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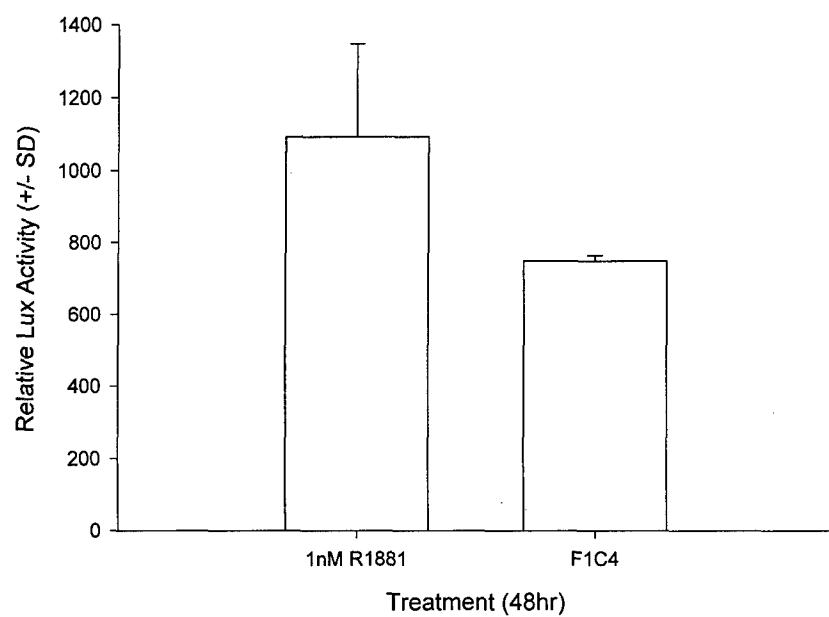


\*  $P < 0.05$

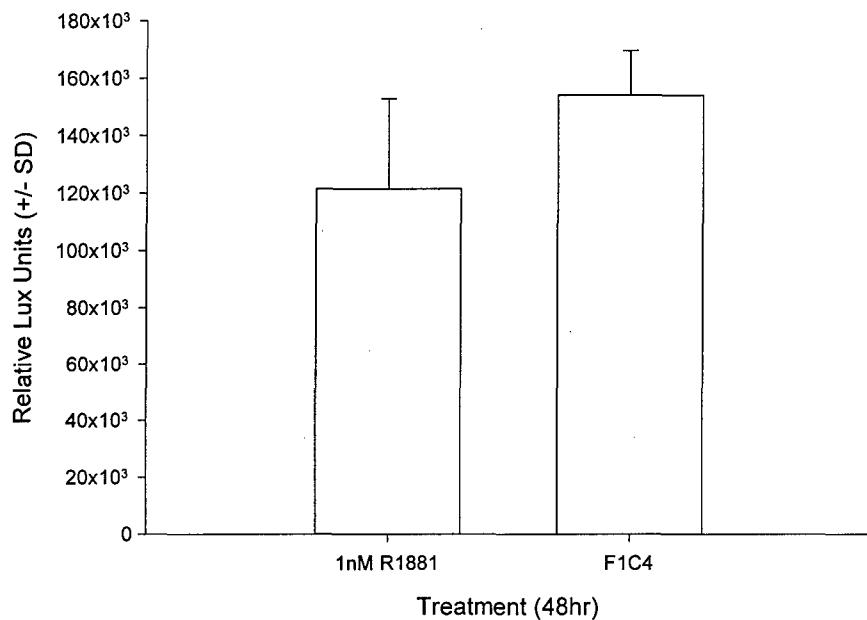
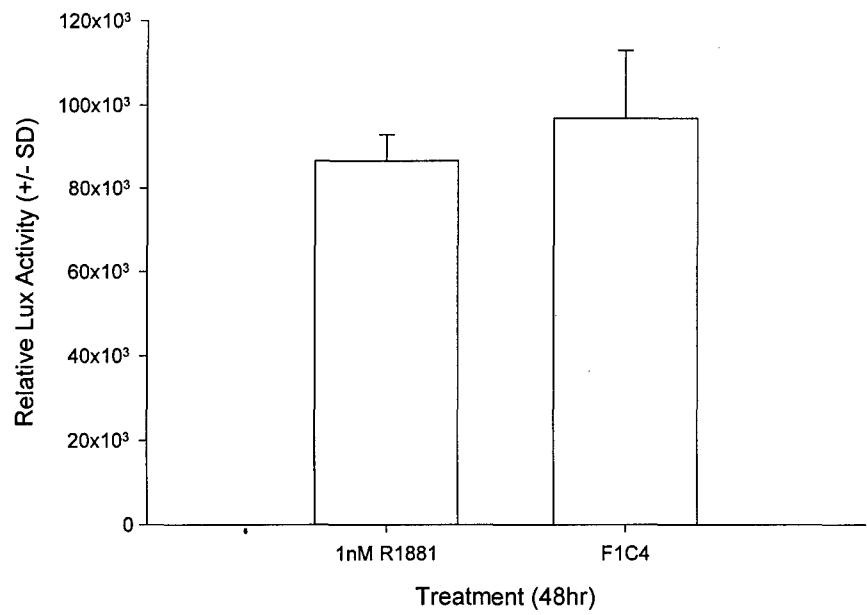
# Appendix 3

**A****pNF $\kappa$ BTKluc Activity in Androgen-Sensitive LNCaP Cells**

\* statistically significantly different ( $P < 0.05$ )

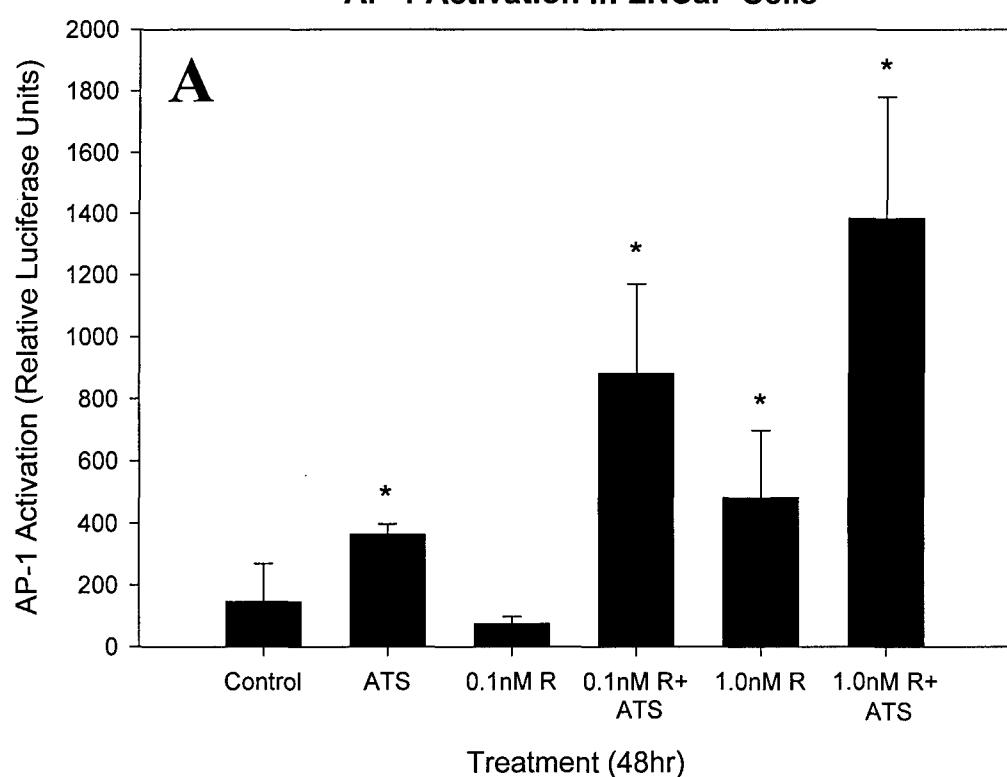
**B****pmutNF- $\kappa$ BTKluc Activity in Androgen-Sensitive LNCaP Cells**

# Appendix 4

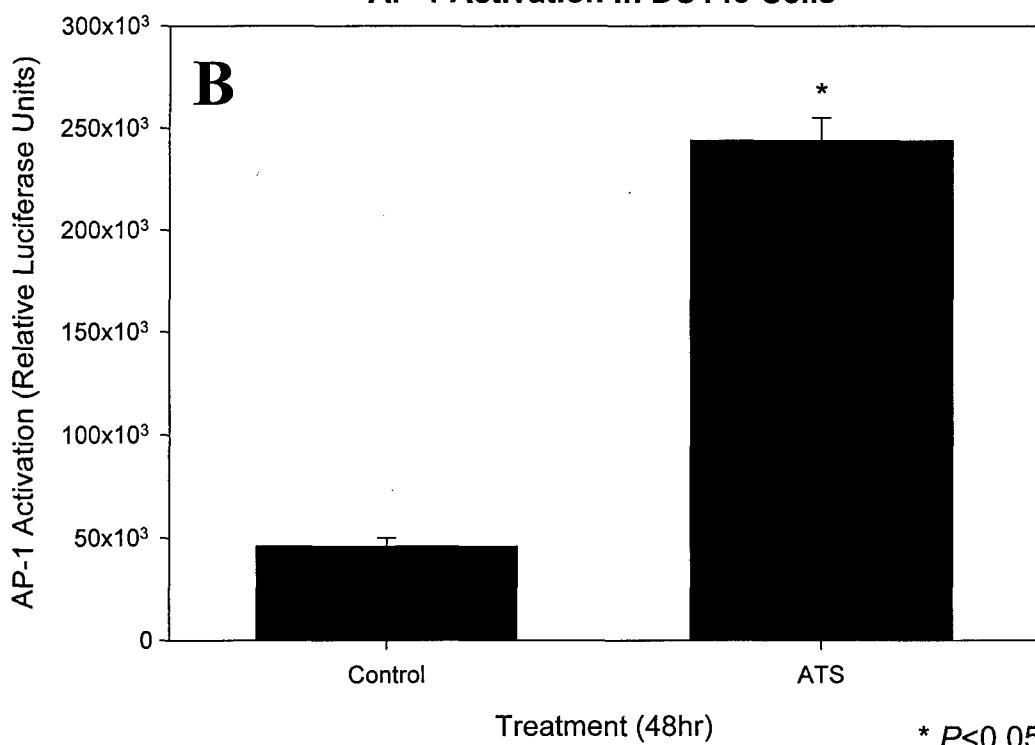
**A****pNF $\kappa$ BTKluc Activity in Androgen-Independent Du145 Cells****B****pmutNF- $\kappa$ BTKluc Activity in Androgen-Independent Du145 Cells**

# Appendix 5

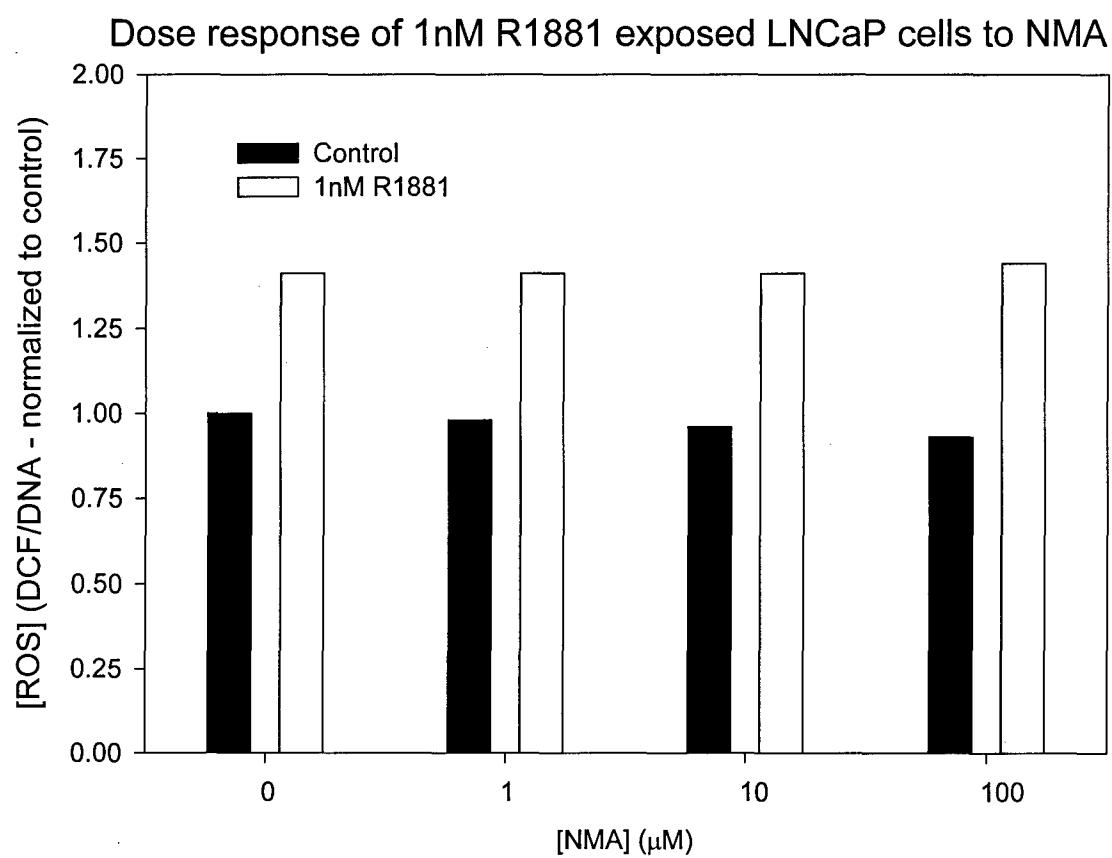
## AP-1 Activation in LNCaP Cells



## AP-1 Activation in DU145 Cells



# Appendix 6



**Appendix 7****A.**

Whole cell Bcl-2



Mitochondrial Bcl-2



Mitochondrial Bax



Lane 1: Control

Lane 2: 0.1nM R1881

Lane 3: 1.0nM R1881

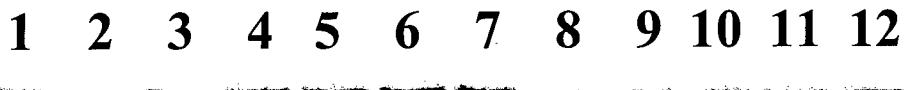
Lane 4: 20uM ATS

Lane 5: 20uM ATS + 0.1nM R1881

Lane 6: 20uM ATS + 1.0nM R1881

**B.**

Whole cell Bak

**C.**

Actin

LNCaP Cells

Lane 1: Control

Lane 2: 0.1nM R1881

Lane 3: 1.0nM R1881

Lane 4: 20uM ATS

Lane 5: 20uM ATS + 0.1nM R1881

Lane 6: 20uM ATS + 1.0nM R1881

DU145 Cells

Lane 7: Control

Lane 8: 0.1nM R1881

Lane 9: 1.0nM R1881

Lane 10: 20uM ATS

Lane 11: 20uM ATS + 0.1nM R1881

Lane 12: 20uM ATS + 1.0nM R1881

Thompson, Todd A.

## BIBLIOGRAPHY

Publications and meeting abstracts resulting from this research effort include:

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Thompson, T.A., Johnson, J.A., Johnson, D.A., and Wilding, G.W. Butylated hydroxyanisole reverses the growth modulatory activity of androgens in LNCaP human prostate carcinoma cells. *Proc. Am. Assoc. Cancer Res.* 42, 2001 (A1888).

Church, D.R., Ripple, M.O., Kolb, R.M., Thompson, T.A., and Wilding, G. Androgen induces a change in the components of the AP-1 transcription factor complex in LNCaP human prostate carcinoma cells. *Proc. Am. Assoc. Cancer Res.* 42, 2001 (A4722).

Thompson, T.A. and Wilding, G. The food additives butylated hydroxyanisole and butylated hydroxytoluene are antiandrogenic. Presented at the Hormonal Carcinogenesis Gordon Research Conference, 2001.

## PERSONNEL

Personnel who received pay from this research effort include:

Thompson, Todd A.

Thomas, James P.



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
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FORT DETRICK, MARYLAND 21702-5012

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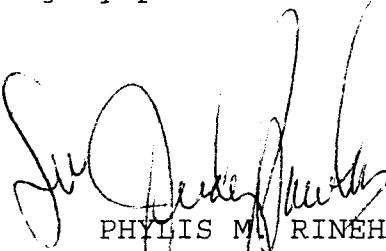
8 Jan 2003

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FOR THE COMMANDER:



PHYLIS M. RINEHART  
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